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(54) Title: AGENT FOR REVERSAL OF DRUG RESISTANCE IN MYCOBACTERIUM TUBERCULOSIS

(57) Abstract: Multidrug resistance to anti-tuberculous drugs poses threat in the treatment of tuberculosis. These strains are resistant to at least two first line anti-tuberculous drugs such as INH and rifampicin. Frequently, such MDR strains show resistance to all commonly used first-line agent i.e. INH, rifamcpicin, streptomycin, ethambutol and pyrazinamide. Isoniazid is the most widely used anti-tuberculous drug. Resistance to isoniazid can occur by increased expression in inhA or by mutations that lower the enzyme's affinity to NADH. Mutations in katG, which encodes catalase peroxidase, is the most common source of resistance. Another mechanism of isoniazid resistance occurs by defects in NADH dehydrogenase (Ndh) of the respiratory chain. Increases expression of AphC has been suggested as another mechanism of INH resistance in mycobacteria. The present invention overcomes INH resistance by the use of penicillins with INH. According to present invention, penicillins when used in effective amount, reduces the MIC of INH in multi-drug resistant strains of M. tuberculosis. The improved INH sensitivity, as brought by the present invention, falls within the range which can be exploited for effective therapeutic intervention.

1. AGENTS FOR REVERSAL OF DRUG RESISTANCE IN MYCOBACTERIUM TUBERCULOSIS.

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3. The following specification particularly describes the nature of this invention and the manner in which it is to be performed.

FIELD OF THE INVENTION

The present invention provides agents for reversal of INH resistance in Mycobacterium tuberculosis. According to present invention, penicillins reverses resistances of Mycobacterium to INH.

THE BACKGROUND

Isoniazid is the most widely used anti-tuberculous drug. It is one of the main therapeutic agent. The strains of mycobacterium are usually inhibited at the concentration of 0.2 mcg/ml or less. When a particular strain is not inhibited by INH concentration of 1.0 mcg/ml, it is labeled as resistant strain. Inh-A, NADH-dependent enocyl acyl carrier protein reductase is the primary target for this drug (Miesel L et al., 1998). A reactive form of isoniazid inhibits inh-A by reacting with the NADH cofactor to the enzyme active site forming a covalent adduct (isonicotinic acyl NADH) that is apt to bind with high affinity.

There are more than one mechanisms by which mycobacterium becomes resistant to INH. Resistance to isoniazid can occur by increased expression of inh-A or by mutations that lower the enzyme's affinity to NADH (Miesel Let al., 1998). Both of these resistance mechanisms are observed in 30% of clinical tuberculosis isolates. Mutations in katG, which encodes catalase peroxidase, is the most common source for resistance (Miesel L et al., 1998).

Another mechanism of isoniazid resistance occurs by defects in NADH dehydrogenase (Ndh). of the respiratory chain. Genetic studies indicate that Ndh mutations confer resistance by lowering the rate of NADH oxidation and increasing the intracellular NADH/NAD+ ratio.

Increased expression of *AhpC* has been suggested as another mechanism of INH resistance in mycobacteria (Telenti A et al., 1997; Dhandayuthapani S et al., 1996; Wilson TM et al., 1996).

For the treatment of INH resistant tuberculosis, including multidrug resistant tuberculosis drugs like ciprofloxacin, Kanamycin, Prothionamide etc. are used along with other primary drugs.

Infection caused by resistant organisms is on increase and is becoming a major problem. Efforts are made to discover new regimens, new drugs to minimize the incidence of the problem as well as treat it effectively.

The management of resistant infection involve use of drugs to which organisms are sensitive.

Penicillin is the oldest antibiotic available and in use even to-day. It has been evaluated for management of various infections including mycobacterial infections (US Public Health Service General Research Support, 1973). Use of penicillin alone had not been found to be therapeutically effective in the treatment of tuberculosis. MIC of penicillins against mycobacterium is significantly high. It is not possible to achieve therapeutic concentration of penicillins required to treat infection effectively,

The attempts have been made to overcome this problem by combining penicillins with beta-lactamase inhibitors. One such study evaluated ampicillin in combination with a beta-lactamase inhibitor (Clavulanic acid, sulbactam, BL-P2013 or BL-P2090) in a ratio 1:1 in a study by Sorg TB et al., 1987. MIC of ampicillin alone was 32 mcg/ml, but when combined with Clavulanic acid it was 4 mcg /ml. Clavulanic acid-ampicillin combination was the most active combination with MIC_{90} of 11 µM. Use of Beta-lactamase inhibitors alone were ineffective at highest concentrations tested.

Other studies showed ampicillin-sulbactam to have a bactericidal effect at very high concentrations (15 mcg ampicillin per ml) in vitro on exponential phase cultures of M. tuberculosis, thereby indicating its usefulness only in early period of anti-tuberculous treatment (Herbert D et al., 1996). However, they are unlikely to be effective as sterilizing drugs helping to kill persisting lesional bacilli. Studies by Cynamon CH et al., 1983 and Wong CS, 1988 reveal that Amoxycillin-Clavulanic acid combination was bactericidal at concentrations of Amoxycillin 4 mcg/ml and Clavulanic acid 2 mcg/ml in 14 of 15 strains tested. Even for this, dose required is very high.

All of the above way was done for strains sensitive to drugs. The above use of beta-lactamase inhibitor-penicillin combination did not focus on multi-drug resistant strains of M. tuberculosis. Penicillin has not been tried to reverse the resistance of mycobacterium to INH so far. It has also not been successfully used in management of resistant mycobacterial infection.

The combination of Amoxycillin and a beta-lactamase inbibitor, Clavulanic acid was evaluated in a clinical setting for the treatment of multidrug-resistant M. tuberculosis without success (Nadler JP et al, 1991). In another study by Nakagawa et. al.(1999), the combination of a beta-lactamase inhibitor and penicillin was evaluated clinically in 4 patients with MDR tuberculosis. 2 patients discontinued therapy on account of diarrhoea and in other two, treatment was continued, but MDR-TB remained positive during the remaining period of therapy.

Addition of ethambutol significantly reduces the MIC of Amoxycillin for isolates from 16 mg/L to $\leq$ 0.5 mg/L (Abate G et al., 1998).

Alternative approaches have been use of alternative drugs like clarithromycin to overcome the problem of multi-drug resistance (Cavalieri SJ et al., 1995). Ciprofloxacin has been used in newly diagnosed cases of pulmonary tuberculosis (Frederick A et al., 1997). However, emergence of resistance to ciprofloxacin against MDR strains has aroused concern (Fattorini L ae tal., 1999).

One attempt was made to increase the susceptibility of multi-drug resistant M. tuberculosis to anti-tuberculous drugs (Jagannath C etal., 1995). The study involved used of ethambutol and dimethyl sulfoxide. Pre-exposure to these compounds enhanced the activity of anti-tuberculous drugs against multi-drug resistant strains of M. tuberculosis.

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SUMMARY OF THE INVENTION

The present invention overcomes the problem of INH resistance by penicillins. According to present invention, penicillins are found to cause reduction in MIC of INH against multi-drug resistant strains of Mycobacterium tuberculosis, and making them sensitive to INH. The strains of mycobacterium not inhibited by INH at concentration of 1.0 mcg/ml by agar plate method gets inhibited by INH when 1.0 to 2.0 mcg/ml of penicillins is added to it.

DESCRIPTION OF THE INVENTION

The present invention uses penicillins to overcome the problem of INH resistance. This has been done by using penicillins in combination with INH.

Penicillins on its own, does not have activity against M. tuberculosis at a dose used or can be used. But when it is combined with INH, it improves sensitivity profile (MIC) of INH. The strains which are resistant to INH becomes sensitive to INH. The strain is known as resistant if it is not inhibited at 1.0 mcg/ml.

For the purpose of demonstrating reversal of mycobacterial resistance to INH, an agar plate method has been used.

EXAMPLE :

I. Preparation of LJ media with combination drugs (using penicillin and INH):

Stock solution of Isoniazid and penicillin was prepared so as to obtain a strength of 10,000/ml.

Three concentrations of INH were used (0.1, 0.2 and 1.0 mcg/ml) along with penicillin. Appropriate solution was mixed with LJ medium so as to give the desired final concentrations as given below. All the flasks and tubes were marked before use, with the drug symbol and final concentration they obtain.

Note: Figures with underline are for Isoniazid
Figures with bold are for penicillin

INH 0.1 + Penicillin:

Antibiotic conc. (mcg/ml)	*Antibiotic Dist. Water (ml)*	*Amount transferred (ml)*	+	*LJ Medium (ml)*	*Final conc. (mcg/ml)*
10,000 **10,000**	0.01 **0.025** 9.965	1.0	+	99	0.1 + **0.25**
	0.01 **0.05** 9.94	1.0	+	99	0.1 + **0.5**
	0.01 **0.1** 9.89	1.0	+	99	0.1 + **1**
	0.01 **0.2** 9.79	1.0	+	99	0.1 + **2**

INH 0.2 + Penicillin:

Antibiotic conc. (mcg/ml)	*Antibiotic Dist. Water (ml)*	*Amount transferred (ml)*	+	*LJ Medium (ml)*	*Final conc. (mcg/ml)*
10,000 **10,000**	0.02 **0.025** 9.955	1.0	+	99	0.2 + **0.25**
	0.02 **0.05** 9.93	1.0	+	99	0.2 + **0.5**
	0.02 **0.1** 9.88	1.0	+	99	0.2 + **1**
	0.02 **0.2** 9.78	1.0	+	99	0.2 + **2**

INH 1.0 + Penicillin:

Antibiotic conc. (mcg/ml)	*Antibiotic Dist. Water (ml)*	*Amount transferred (ml)*	+	*LJ Medium (ml)*	*Final conc. (mcg/ml)*
10,000 **10,000**	0.1 **0.025** 9.875	1.0	+	99	1.0 + **0.25**
	0.1 **0.05** 9.85	1.0	+	99	1.0 + **0.5**
	0.1 **0.1** 9.8	1.0	+	99	1.0 + **1**
	0.1 **0.2** 9.7	1.0	+	99	1.0 + **2**

II. Evaluation of various penicillins for reversal of INH resistance of mycobacterium.

II A. Cloxacillin:

Ten strains (clinical isolates) of M. tuberculosis with various sensitive and resistant patterns to first-line anti-tuberculous drugs were evaluated using varying concentrations of INH (0.1, 0.2 and1.0) and Cloxacillin (0.25, 0.5, 1.0 and 2.0) combination and MIC were determined for each group.

Three groups were made: One group had INH 0.2 and Cloxacillin and in this, 4 concentrations using 0.25, 0.5, 1.0 and 2.0 were used to determine MIC. In another group, INH 0.2 and Cloxacillin was used and this as well, 4 concentrations (0.25, 0.5, 1.0 and 2.0) were used to determine MIC. In the third group as well, INH 1.0 and Cloxacillin were taken and again similar 4 concentrations were used. So in each group, 4 LJ bottles for each M. tuberculosis strain was used. For one strain, in all, 12

bottles were used to determine MIC. The experiments were repeated more than once for consistency.

At the end of period, each bottles were observed for presence or absence of growth.

The results of the 10 strains are shown in the table below:

Clinical isolate	Control	INH 0.1 + Cloxacillin				INH 0.2 + Cloxacillin				INH 1.0 + Cloxacillin			
		0.25	0.5	1.0	2.0	0.25	0.5	1.0	2.0	0.25	0.5	1.0	2.0
I. SENSITIVE TO INH, RIFA, SM AND EMB													
2328	+++	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG
2981	+++	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG
2903	+++	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG
II. RESISTANT TO INH ONLY													
3008	+++	++	NG	NG	NG	++	NG	NG	NG	++	NG	NG	NG
III. RESISTANT TO INH, RIFA													
1970	+++	+++	NG	NG	NG	+++	NG	NG	NG	+++	NG	NG	NG
IV. RESISTANT TO INH, RIFA, EMB													
2761	+++	+++	++		NG	+++	++	NG	NG	+++	NG	NG	NG
V. RESISTANT TO INH, RIFA, EMB, SM													
2924	+++	++	++	NG	NG	++	++	NG	NG	++	NG	NG	NG
2509	+++	+++	++	++	NG	+++	+++	NG	NG	+++	++	NG	NG
3154	+++	+++	+++	+	NG	+++	+++	+	NG	+++	++	NG	NG
2171	+++	+++	+++	++	NG	+++	+++	++	NG	+++	+++	NG	NG

Notes:

- NG: No growth
- All the drug concentrations are in μg/ml
- INH-Isoniazid, RIFA-Rifampicin, EMB-Ethambutol, SM-Streptomycin

The findings reveal that strains resistant to INH at more than 1.0 mcg/ml becomes sensitive to INH at 0.1 mcg/ml, when Cloxacillin 2.0 mcg/ml is used along with it. All strains become sensitive to INH 1.0 mcg/ml in presence of 1.0 mcg/ml of Cloxacillin.

The change in sensitivity profile is dependent on amount of INH + Cloxacillin.

Findings also suggests that amount of INH and Cloxacillin required is more if organism is resistant to more drugs. Findings also suggests that Cloxacillin concentration is more important than INH concentration, since 2.0 mcg/ml of Cloxacillin is effective for all strains irrespective of INH concentration.

Example II B. Amoxycillin:

Similarly, Amoxycillin was also evaluated. The strength of amoxycillin evaluated were 1.0 and 2.0 mcg/ml

Patient ID	Control	INH 0.2 + Amoxycillin		INH 0.4 + Amoxycillin		INH 1.0 + Amoxycillin	
		1.0	2.0	1.0	2.0	1.0	2.0
I. Strains resistant to INH only, but sensitive to RIFA, SM and EMB							
1435	+++	NG	NG	NG	NG	NG	NG
1750	+++	++	NG	NG	NG	NG	NG
2135	+++	NG	NG	NG	NG	NG	NG
2139	+++	++	++	NG	NG	NG	NG
II. Strains resistant to INH, RIFA and EMB, but sensitive to SM							
1441	+++	NG	NG	NG	NG	NG	NG
1890	+++	++	++	++	++	++	NG
III. Strains resistant to INH, RIFA, SM and EMB							
1574	+++	++	++	++	++	NG	NG
1366	+++	++	++	++	NG	++	NG
1856	+++	++	++	++	NG	++	NG
2160	+++	++	++	NG	NG	NG	NG

Notes:

- NG: No growth
- All the drug concentrations are in μg/ml
- INH-Isoniazid, RIFA-Rifampicin, EMB-Ethambutol, SM-Streptomycin

The data reveals that findings obtained with Amoxycillin are identical to that seen with Cloxacillin.

1. For strains resistant to INH only, MIC appears to be 1 mcg/ml.

2. For strains resistant to INH, Rifampicin and ethambutol, MIC ranges between 1 to 2 mcg/ml.

3. For strains resistant to all 4 drugs, MIC of 2 and higher may be required.

Example II C. Combination of penicillin:

Cloxacillin is a narrow spectrum penicillin with more effect on organism producing beta-lactamase and/or penicillinase, while Amoxycillin is a broad spectrum penicillin but has no activity on organisms producing beta-lactamase or penicillinase. To find out whether one or the other is more useful, same clinical isolates against which Amoxycillin was evaluated were evaluated with various concentrations of combination of Amoxycillin and Cloxacillin.

The combinations used has Amoxycillin and Cloxacillin in ratio of 1:1, 1:2 and 1:4 respectively.

Patient ID	Control	INH 0.2 + Amox-Clox.(1:1)		INH 0.4 + Amox-Clox.(1:1)		INH 1.0 + Amox-Clox.(1:1)	
		1.0	2.0	1.0	2.0	1.0	2.0
I. Strains resistant to INH RIFA, SM and EMB							
1574	+++	++	++	NG	NG	NG	NG
2160	+++	++	++	++	NG	++	NG
II. Strains resistant to INH, but sensitive to RIFA EMB, SM							
1435	+++	NG	NG	NG	NG	NG	NG
1750	+++	++	++	++	NG	++	NG
2135	+++	++	NG	NG	NG	NG	NG
2139	+++	++	++	++	NG	NG	NG
III. Strains resistant to INH, RIFA, but sensitive to SM and EMB							
1804	+++	++	++	++	++	++	NG
IV. Strains sensitive to SM but resistant to RIFA, INH and EMB							
1441	+++	++	++	NG	NG	NG	NG
1982	+++	++	++	++	++	++	NG

Notes:

- NG: No growth
- All the drug concentrations are in μg/ml
- INH-Isoniazid, RIFA-Rifampicin, EMB-Ethambutol, SM-Streptomycin

Patient ID	Control	INH 0.2 + Amox-Clox.(1:2)		INH 0.4 + Amox-Clox.(1:2)		INH 1.0 + Amox-Clox.(1:2)	
		1.0	2.0	1.0	2.0	1.0	2.0
I. Strains resistant to INH only, but sensitive to RIFA, SM and EMB							
2135	+++	++	NG	NG	NG	NG	NG
1750	+++	++	++	NG	NG	NG	NG
1435	+++	NG	NG	NG	NG	NG	NG
2139	+++	++	++	++	NG	NG	NG
II. Strains resistant to INH, RIFA and EMB, but sensitive to SM							
1441	+++	NG	NG	NG	NG	NG	NG
1982	+++	++	NG	+	NG	NG	NG
III. Strains resistant to INH, RIFA, SM and EMB							
1574	+++	++	NG	NG	NG	NG	NG
2160	+++	++	++	++	++	NG	NG
IV. Strains resistant to INH, RIFA but sensitive to SM and EMB							
1804	+++	++	++	++	++	++	NG

Notes:

- NG: No growth
- All the drug concentrations are in μg/ml
- INH-Isoniazid, RIFA-Rifampicin, EMB-Ethambutol, SM-Streptomycin

Patient ID	Control	INH 0.2 + Amox-Clox.(1:4)		INH 0.4 + Amox-Clox.(1:4)		INH 1.0 + Amox-Clox.(1:4)	
		1.0	2.0	1.0	2.0	1.0	2.0
I. Strains resistant to INH, RIFA, SM and EMB							
1574	+++	++	++	NG	NG	NG	NG
2160	+++	++	NG	NG	NG	NG	NG
II. Strains resistant to INH, RIFA and EMB, but sensitive to SM							
1441	+++	NG	NG	NG	NG	NG	NG
1890	+++	++	++	++	NG	++	NG
1982	+++	++	++	++	NG	NG	NG
III. Strains resistant to INH, but sensitive to RIFA, SM and EMB							
1435	+++	NG	NG	NG	NG	NG	NG
1750	+++	NG	NG	NG	NG	NG	NG
2135	+++	NG	NG	NG	NG	NG	NG
2139	+++	++	++	NG	NG	NG	NG
IV. Strains resistant to INH and RIFA, but sensitive to SM and EMB							
1804	+++	++	++	++	++	++	NG

Notes:

- NG: No growth
- All the drug concentrations are in μg/ml
- INH-Isoniazid, RIFA-Rifampicin, EMB-Ethambutol, SM-Streptomycin

The findings show that for reversal of resistance to INH, all penicillins have identical effect when used alone or in combination with each other.

The above example clearly demonstrates that penicillins reverses the resistance of mycobacterium for INH. The examples also indicates the amount of penicillins required to lower the MIC of INH for INH resistant mycobacterium. The amount required is easily achievable in clinical settings without any adverse effects.

I claim:

1. Penicillin reverses resistance of mycobacterium to INH.

2. Penicillins, as claimed in claim 1 can be a broad spectrum penicillin or a narrow spectrum penicillin.

3. Penicillin, as claimed in claim 1 and 2 can be any single penicillin or combination of more than one penicillins.

4. Penicillin, as claimed in claim 1 to 3 can have activity against beta-lactamase producing organism.

5. Penicillin, as claimed in claim 1 to 3 can have activity against penicillinase producing organism.

6. Penicillins, as claimed in claim 1 to 3 can be selected from the group of penicillin comprising Benzylpenicillin, Phenoxymethylpenicillin, Propicillin, Clemizole penicillin, Procaine penicillin, Phenethicillin, Hetacillin, Metampicillin, Pivampicillin, Cyclacillin, Epicillin, Pevmecillinam, Apalcillin, Aspoxicillin, Temocillin, Methicillin, Oxacillin, Nafcillin, Ampicillin, Amoxycillin, Carbenicillin, Ticarcillin, Azlocillin, Mezlocillin, Piperacillin, Cloxacillin, Dicloxacillin, Lenampicillin, Bacampicillin, Azidocillin, Talampicillin, Mecillinam, Flucloxacillin, Aztreonam, Imipenem, Meropenem and the like.

7. Mycobacterium, as claimed in claim 1 can be Mycobacterium tuberculosis.